be used, but other amino acids are preferred. Typical peptides include GRPRTSSFAEG (SEQ ID NO:1), RPRAATC (SEQ ID NO:2) or functional equivalents thereof. The peptide is a substrate for measuring PKB activity.

Replace the paragraph on page 11, lines 22-35, with the following new paragraph:

Mono Q chromatography was performed as described (Burgering et al., 1995), except that the buffer also contained 1 mM EGTA, 0.1 mM sodium orthovanadate and 0.5% (w/v) Triton X-100. Two PKBα antibodies were raised in rabbits against the C-terminal peptide FPQFSYSASSTA (SEQ ID NO:3) and bacterially expressed PH domain of PKBα. The C-terminal antibody was affinity purified (Jones et al., 1991). The activity of PKB towards Crosstide is threefold higher than its activity towards histone H2B and 11-fold higher than its activity towards myelin basic protein, the substrates used previously to assay PKB. Other experimental details and units of protein kinase activity are given in Fig 1.

Replace the paragraph on page 15, lines 5-22, with the following new paragraph:

To identify the insulin-stimulated protein kinase that inhibits GSK3 in the presence of rapamycin and PD 98059, L6 myotubes were incubated with both compounds and stimulated with insulin. The lysates were then chromatographed on Mono Q and the fractions assayed with "Crosstide" (GRPRTSSFAEG)(SEQ ID NO:1), a peptide corresponding to the sequence in GSK3 surrounding the serine (underlined) phosphorylated by MAPKAP kinase-1 and $p70^{S6k}$ (Ser 21 in GSK3-α (Sutherland et al., 1994) and Ser 9 in GSK3-β (Sutherland et al., 1993)). Three peaks of Crosstide kinase activity were detected, which were absent if insulin stimulation was omitted or if the cells were first preincubated with the PI 3-kinase inhibitor wortmannin (Fig 2a). Wortmannin (Cross et al., 1994 and

Welsh et al., 1994), and the structurally unrelated PI 3-kinase inhibitor LY 294002 (ref 19); (Fig 1b), both prevent the inhibition of GSK3 by insulin.

Replace the paragraph on page 25, lines 11-34, with the following new paragraph:

Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of PKBα causing partial activation. Ser-473 of PKBα lies in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr (SEQ ID NO:11) found to be conserved in a number of protein kinases that participate in signal transduction pathways (Pearson et al. 1995). In order to identify the Ser-473 kinase(s) we therefore chromatographed rabbit skeletal muscle extracts on CM-Sephadex, and assayed the fractions for protein kinases capable of phosphorylating a synthetic peptide corresponding to residues 465 to 478 of PKBα. These studies identified an enzyme eluting at 0.3 M NaCl which phosphorylated the peptide 465-478 at the residue equivalent to Ser-473 of PKBα. The Ser473 kinase co-eluted from CM-Sephadex with MAP kinase-activated protein (MAPKAP) kinase-2, (Stokoe et al., 1992) which is a component of a stress and cytokine-activated MAP kinase cascade (Rouse et al., 1994; Cuenda et al., 1995). The Ser-473 kinase continued to cofractionate with MAPKAP kinase-2 through phenyl-Sepharose, heparin-Sepharose, Mono S and Mono Q and was immunoprecipitated quantitatively by an anti-MAPKAP kinase-2 antibody (Gould et al., 1995) demonstrating that MAPKAP kinase-2 was indeed the Ser-473 kinase we had purified.

Replace the paragraph on page 33, line 23 through page 34, line 8, with the following new paragraph:

Thr-308 is located in subdomain VIII of the kinase catalytic domain, nine residues upstream of the conserved Ala-Pro-Glu motif, the same position as activating phosphorylation sites found in many other protein kinases. However, Ser-473 is located C-terminal to the

catalytic domain in the consensus sequence Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr (SEQ ID NO:11) which is present in several protein kinases that participate in growth factor-stimulated kinase cascades, such as p70 S6 kinase, PKC and p90rsk (Pearson et al., 1995). However, it is unlikely that a common protein kinase phosphorylates this motif in every enzyme for the following reasons. Firstly, phosphorylation of the equivalent site in p70 S6 kinase is prevented by the immunosuppressant drug rapamycin (Pearson et al., 1995) which does not prevent the activation of PKBα by insulin (Cross et al., 1995) or it's phosphorylation at Ser-473 (D. Alessi, unpublished work). Secondly, the equivalent residue in protein kinase C is phosphorylated constitutively and not triggered by stimulation with growth factors (Tsutakawa et al., 1995).

Replace the paragraph on page 34, line 10 through page 35, line 9, with the following new paragraph:

MAPKAP kinase-2 is a component of a protein kinase cascade which becomes activated when cells are stimulated with interleukin-1 or tumour necrosis factor or exposed cellular stresses (Rouse et al., 1994; Cuenda et al., 1995). MAPKAP kinase-2 phosphorylates PKBα stoichiometrically at Ser-473 (Fig 11) and this finding was useful in establishing the role of Ser473 phosphorylation in regulating PKBα activity. However, although MAPKAP kinase-2 activity is stimulated to a small extent by insulin in L6 cells, no activation could be detected in 293 cells in response to insulin or IGF-1. Moreover, exposure of L6 cells or 293 cells to a chemical stress (0.5 mM sodium arsenite) strongly activated MAPKAP kinase-2 (D. Alessi, unpublished work) as found in other cells (Rouse et al., 1994; Cuenda et al., 1995), but did not activate PKBα at all. Furthermore, the drug SB 203580 which is a specific inhibitor of the protein

kinase positioned immediately upstream of MAPKAP kinase-2 (Cuenda et al., 1995), prevented the activation of MAPKAP kinase-2 by arsenite but had no effect on the activation of PKBα by insulin or IGF-1. Finally, the activation of PKBα was prevented by wortmannin (Figs 6 and 9), but wortmannin had no effect on the activation of MAPKAP kinase-2 in L6 or 293 cells. It should also be noted that the sequence surrounding Ser-473 of PKBα (HFPQFSY) (SEQ ID NO:12) does not conform to the optical consensus for phosphorylation by MAPKAP kinase-2 which requires Arg at position n-3 and a bulky hydrophobic residue at position n-5, (where n is the position of the phosphorylated residue). The Km for phosphorylation of the synthetic peptide comprising residues 465-478 is nearly 100-fold higher than the Km for the standard MAPKAP kinase-2 substrate peptide (data not shown). It is therefore unlikely that MAPKAP kinase-2 mediates the phosphorylation of Ser-473 in vivo.

Replace the paragraph on page 37, lines 10-31, with the following new paragraph:

The activation of PI 3-kinase is essential for many of the effects of insulin and growth factors, including the stimulation of glucose transport, fatty acid synthesis and DNA synthesis, protection of cells against apoptosis and actin cytoskeletal rearrangements (reviewed in Carpenter et al., 1996). These observations raise the question of whether PKBα mediates any of these events by phosphorylating other proteins. To address this issue we characterised the substrate specificity requirements of PKBα. We find that the optimal consensus sequence for phosphorylation by PKBα is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd (SEQ ID NO:4), where Yaa and Zaa are small amino acids (other than glycine) and Hyd is a large hydrophobic residue (such as Phe or Leu). We also demonstrate that PKBα phosphorylates histone H2B (a

substrate frequently used to assay PKBα in vitro) at Ser-36 which lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (SEQ ID NO:4). These studies identified a further PKBα substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe)(SEQ ID NO:6) that, unlike other peptides, is not phosphorylated to a significant extent by either p70 S6 kinase or MAPKAP-K1.

Replace the paragraph on page 38, line 23 through page 39, line 11, with the following new paragraph:

Identification of the residues in histone H2B phosphorylated by PKBα. Currently, three substrates are used to assay PKBα activity in different laboratories, histone H2B, MBP and Crosstide. PKBα phosphorylated Crosstide with a Km of 4 μM and a Vmax of 260 U/mg (Table 7.1 A, peptide 1), histone H2B with a Km of 5 μM and a Vmax of 68 U/mg, and MBP with a Km of 5 μM and a Vmax of 25 U/mg. Thus the Vmax of histone H2B and MBP are 4-fold and 10-fold lower than for Crosstide. In order to identify the residue(s) in histone H2B phosphorylated by PKBα, ^{32}P-labelled histone H2B was digested with trypsin (see Methods) and the resulting peptides chromatographed on a C18 column at pH 1.9. Only one major ^{32}P-labelled peptide (termed T1) eluting at 19.5 % acetonitrile was observed (Fig 17A). The peptide contained phosphoserine (data not shown), its sequence commenced at residue 34 of histone H2B and a single burst of radioactivity occurred after the third cycle of Edman degradation (Fig 17B), demonstrating that PKBα phosphorylates histone H2B at Ser-36 within the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr (SEQ ID NO:7). Thus, like the serine phosphorylated in Crosstide, Ser-36 of histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (SEQ ID NO:5)(where Hyd is a bulky hydrophobic residue, -Phe in Crosstide, Tyr in H2B).

Replace the paragraph on page 39, lines 13-32, with the following new paragraph:

Molecular basis for the substrate specificity of PKBα.

To further characterise the substrate specificity requirements for PKBα, we first determined the minimum sequence phosphorylated efficiently by PKBα by removing residues sequentially from the C-terminal and N-terminal end of Crosstide. Removal of the N-terminal glycine and up to three residues from the C-terminus had little effect on the kinetics of phosphorylation by PKBα (Table 7.1A, compare peptides 1 and 5). However any further truncation of either the N or C-terminus virtually abolished phosphorylation (Table 7.1A, peptides 8 and 9). The minimum peptide phosphorylated efficiently by PKBα (Arg-Pro-Arg-Thr-Ser-Ser-Phe)(SEQ ID NO:9) was found to be phosphorylated exclusively at the second serine residue as expected. Consistent with this finding, a peptide in which this serine was changed to alanine was not phosphorylated by PKBα (Table 7.1A, peptide 7). All further studies were therefore carried out using variants of peptide 5 in Table 7.1A (see below).

Replace the paragraph on page 40, line 29 through page 41, line 8, with the following new paragraph:

Replacement of the Thr situated two residues N-terminal to the phosphorylated serine increased the Km with any amino acid tested (Table 7.1C). Substitution with Ala only increased Km by 2-3 fold, but substitution with other residues was more deleterious and with Asn (a residue of similar size and hydrophilicity to Thr) phosphorylation was almost abolished (Table 7.1C). Replacement of the Ser situated one residue N-terminal to the phosphorylated serine also increased the Km with any amino acid tested, but the effects were less severe than at position n-2 (Table 7.1C). When residues n-2 and n-1 were both changed to Ala, the resulting peptide RPRAASF (SEQ ID NO:13) was phosphorylated by PKBα with a Km only 5-fold higher than RPRTSSF (SEQ ID NO:9). In contrast

the peptides RPRGGSF (SEQ ID NO:15), RPRAGSF (SEQ ID NO:16), and RPRGASF (SEQ ID NO:17) were phosphorylated less efficiently (Table 7.1C).

Replace the paragraph on page 41, lines 21-29, with the following new paragraph:

MAPKAP kinase-1 and p70 S6 kinase phosphorylate the peptides KKKNRTLSVA (SEQ ID NO:18) and KKRNRTLSVA (SEQ ID NO:19) with extremely low Km values of 0.2- 3.3 μM, respectively (Table 7.2). However, these peptides were phosphorylated by PKBα with 50-900 fold higher Km values (Table 7.2A, peptides 1 and 2). The peptide KKRNRTLTV (SEQ ID NO:20), which is a relatively specific substrate for p70 S6 kinase (Leighton et al., 1995) was also phosphorylated very poorly by PKBα (Table 7.2A, peptide 4).

Replace the paragraph on page 41, line 31 through page 42, line 23, with the following new paragraph:

Crosstide is phosphorylated by p70 S6 kinase and MAPKAP kinase-1 with similar efficiency to PKBα ((Leighton et al., 1995); Table 7.2B-peptide 1 and Fig 18). However, truncation of Crosstide to generate the peptide RPRTSSF (SEQ ID NO:9) was deleterious for phosphorylation by MAPKAP-K1 and even worse for p70 S6 kinase (Table 7.2B-peptides 1 and 2 and Fig 18). Moreover, changing the phosphorylated serine in RPRTSSF (SEQ ID NO:9) to threonine increased the Km for phosphorylation by p70 S6 kinase much more than for PKBα and almost abolished phosphorylation by MAPKAP-K1 (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF (SEQ ID NO:13) was phosphorylated by MAPKAP-K1 with essentially identical kinetics to that of PKBα; however phosphorylation by p70 S6 kinase was virtually abolished (Table 7.2B-peptide 4 and Fig 18). Based on these observations we synthesized the peptide RPRAATF (SEQ ID NO:21). This peptide was phosphorylated by PKBα with a Km of 25μM and similar Vmax

to RPRTSSF (SEQ ID NO:9), but was not phosphorylated to a significant extent by either MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig 18). In Fig 18, the protein kinase concentration in the assays towards Crosstide were 0.2 U/ml, and each peptide substrate was assayed at a concentration of 30 μM. Filled bars denote PKBα activity, hatched bars MAPKAP kinase-1 activity, and grey bars p70 S6 kinase activity. The activities of each protein kinase are given relative to their activity towards Crosstide (100). The results are shown ± SEM for two experiments each carried out in triplicate.

Replace the paragraph on page 42, line 25 through page 43, line 3, with the following new paragraph:

Discussion.

We have established that the minimum consensus sequence for efficient phosphorylation by PKBα is Arg-Xaa-Arg-Yaa-Zaa-Ser-Hyd (SEQ ID NO:10), where Xaa is any amino acid, Yaa and Zaa are small amino acids other than glycine (Ser, Thr, Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu)(Table 7.1). The heptapeptide with the lowest Km value was RPRTSSF (SEQ ID NO:9), its Km of 5 μM being comparable to many of the best peptide substrates identified for other protein kinases. The Vmax for this peptide (250 nmoles min-1 mg-1) may be an underestimate because the PKBα was obtained by immunoprecipitation from extracts of IGF-1 stimulated 293 cells over-expressing this protein kinase, and a significant proportion of the PKBα may not have been activated by IGF-1 treatment.

Replace the paragraph on page 43, lines 5-20, with the following new paragraph:

The requirement for arginine residues at positions n-3 and n-5 (where n is the site of phosphorylation) seems important, because substituting either residue with lysine decreases phosphorylation drastically. Serine and threonine residues were preferred at positions n-1 and n-

2, although the Km value was only increased about 5-fold if both of these residues were changed to Ala. Serine was preferred at position n, since changing it to threonine caused a six-fold increase in the Km. The peptide RPRAATF (SEQ ID NO:21), which was phosphorylated with a Km of 25 μM and similar Vmax to RPRTSSF (SEQ ID NO:9), may therefore be a better substrate for assaying PKBα in partially purified preparations, because unlike Crosstide, it contains only one phosphorylatable residue and is not phosphorylated significantly by MAPKAP-K1 or p70 S6 kinase (Table 7.2, Fig 18 and see below).

Replace the paragraph on page 43, lines 22-35, with the following new paragraph:

The Proline at position n-4 was not altered in this study because it was already clear that this residue was not critical for the specificity of PKBα. Residue n-4 is proline in GSK3β but alanine in GSK3α. Both GSK3 isoforms are equally good substrates for PKBα in vitro (Cross et al., 1995), and the peptide GRARTSSFA (SEQ ID NO:22)(corresponding to the sequence in GSK3α) is phosphorylated by PKBα with a Km of 10 μM and Vmax of 230 U/mg (Table 7.1A, peptide 2). Moreover, in histone H2B, the residue located four amino acids N-terminal to the PKBα phosphorylation site is serine (Fig 17). The presence of Glu and Lys at positions n-1 and n-2 may explain why histone H2B is phosphorylated by PKBα with a four-fold lower Vmax than the peptide RPRTSSF (SEQ ID NO:9).

Replace the paragraph on page 44, line 22 through page 45, line 17, with the following new paragraph:

Additional similarities between p70 S6 kinase, MAPKAP-K1 and PKBα include the failure to phosphorylate peptides containing Pro at position n+1 and dislike of a lysine at the same position. This suggests that, in vivo, these kinases are unlikely to phosphorylate the same residues

as MAP kinases (which phosphorylate Ser/Thr-Pro motifs) or protein kinase C (which prefers basic residues C-terminal to the site of phosphorylation). However, the present work has also revealed significant differences in the specificities of these enzymes. In particular MAPKAP-K1 and (to a lesser extent) p70 S6 kinase can tolerate substitution of the Arg at position n-5 by lysine whereas PKBα cannot (see Table 7.1A, Table 7.2A and (Leighton et al., 1995)). MAPKAP-K1 and p70 S6 kinase can also tolerate, to some extent, substitution of Arg at position n-3 by Lys. For example, the peptide KKRNKTLSVA (SEQ ID NO:23) is phosphorylated by MAPKAP-K1 and p70 S6 kinase with Km values of 17 and 34 μM, respectively, as compared to Km values of 0.7 and 1.5 μM for the peptide KKRNRTLSVA (SEQ ID NO:19)(Table 7.2A). In contrast, PKBα does not phosphorylate the peptide KKRNKTLSVA (SEQ ID NO:23)(Table 7.2A) or any other peptide that lacks Arg at position n-3. PKBα and p70 S6 kinase, but not MAPKAP-K1, phosphorylate Thr as well as Ser (Table 7.1A) and can phosphorylate peptides lacking any residue at position n+2 ((Leighton et al., 1995) and Table 7.2A), while PKBα and MAPKAP-K1, but not p70 S6 kinase, can tolerate substitution of both the n-1 and n-2 positions of the peptide RPRTSSF (SEQ ID NO:9) with Ala (Table 7.2B). These differences explain why the peptide RPRAATF (SEQ ID NO:21) is a relatively specific substrate for PKBα.

Replace the paragraph on page 45, lines 19-27, with the following new paragraph:

One of the best peptide substrates for MAPKAP-K1 and p70 S6 kinase (KKRNRTLSVA)(SEQ ID NO:19) was a poor substrate for PKBα (Table 7.2, peptide 2), despite the presence of Arg at positions n-3 and n-5. The presence of Leu at position n-1 and Val at position n+1 are likely to explain the high Km for phosphorylation, because PKBα

prefers a small hydrophilic residue at the former position and a larger hydrophobic residue at the latter position (Tables 7.1 and 7.2).

Replace the paragraph on page 46, lines 5-7, with the following new paragraph:

Monoclonal antibodies recognising the sequence EFMPME (SEQ ID NO:25)(EE) antibodies and the (EQKLISEEDL)(SEQ ID NO:26) c-Myc were purchased from Boehringer (Lewis, UK).

Replace the paragraph on page 46, lines 13-35, with the following new paragraph:

A DNA construct expressing human GSK3B with the EFMPME (SEQ ID NO:25)(EE) epitope tag at the N-terminus was prepared as follows: A standard PCR reaction was carried out using as a template the human GSK3β cDNA clone in the pBluescript SK+ vector and the oligonucleotides

GCGGAGATCTGCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAGAACC (SEQ ID NO:27)

and GCGGTCCGGAACATAGTCCAGCACCAG (SEQ ID NO:28) that incorporate a *bgl* II site (underlined) and a *Bspe* I site (double underlined). A three-way ligation was then set up in which the resulting PCR product was subcloned as a *Bgl*II-*Bspe* I fragment together with the C-terminal Bspe I-Cla I fragment of GSK3β into the Bgl II-Cla I sites of the pCMV5 vector (Anderson et al., 1989). The construct was verified by DNA sequencing and purified using the Quiagen plasmid Mega kit according to the manufacturer's protocol. The c-Myc GSK3, BA9 construct encodes GSK3β in which Ser-9 is mutated to Ala and possesses a c-myc epitope tag at the C-terminus and was prepared as described in Sperber et al., 1995. The c-Myc GSK3β A9 gene was then subcloned into Xba I/EcoRI sites of the pCMV5 eukaryotic expression vector.

Replace the paragraph on page 49, lines 3-30, with the following new paragraph:

Example 10: basic assay for identifying agents which affect the activity of PKB.

A 40 μl assay mix was prepared containing protein kinase (0.2U/ml) in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, protein kinase substrate (30μM), and the indicated concentration of Ro-318220 or GC 109203X (test inhibitors). After incubation on ice for 10 min the reaction was started by the addition of 10 μl of 50mM magnesium acetate and 0.5 mM [γ^{32}P]ATP (100-200 cpm/pmol). For the assay of mixed isoforms of PKC 20 μM diacylglycerol, 0.5 mM $CaCl_2$, and 100 μM phosphatidylserine were also present in the incubations. The assays were carried out for 15 min at 30°C, then terminated and analysed as described (Alessi 1995). One unit of activity was that amount of enzyme that catalysed the phosphorylation of 1nmol of substrate in 1 min. The final concentration of DMSO in easy assay was 1% (by vol). This concentration of DMSO does not inhibit any of these enzymes. Mixed isoforms of PKC were assayed using histone H1 as substrate, while MAPKAP-K1β and p70 S6 kinase were assayed using the peptide KKRNRTLSVA (SEQ ID NO:19)(Leighton 1995). Protein kinase B was assayed with the peptide GRPRTSSFAEG (SEQ ID NO:1)[9] and MAPKAP-K2 was assayed with the peptide KKLNRTLSVA (SEQ ID NO:57)(Stokoe 1993). p42 MAP kinase was assayed using MBP, and MAPKK-1, and c-Raf1 were assayed as described in Alessi 1995.

Replace the paragraph on page 51, lines 1-22, with the following new paragraph:

General Materials and Methods. Tissue culture reagents, myelin basic protein (MBP), microcystin-LR, and IGF-1 were obtained from Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk (Bagsvaerd, Denmark), phosphate

free Dulbecco's minimal essential medium (DMEM) from ICN (Oxon, UK), Protein G-Sepharose and CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and fluroisothiocyanate-labelled antimouse IgG from goat from Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were raised in sheep against the peptides RPHFPQFSYSASGTA (SEQ ID NO:29)(corresponding to the last 15 residues of rodent PKBα) and MTSALATMRVDYEQIK (SEQ ID NO:30)(corresponding to residues 352 to 367 of human MAPKAP kinase-2) and affinity purified on peptide-CH-Sepharose. Monoclonal HA antibodies were purified from the tissue culture medium of 12CA5 hybridoma and purified by chromatography on Protein G Sepharose. The peptide RPRHFPQFSYSAS (SEQ ID NO:31), corresponding to residues 465-478 of PKBα, was synthesized on an Applied Biosystems 430A peptide synthesizer. cDNA encoding residues 46-400 of human MAPKAP kinase-2 was expressed in E.coli as a glutathione S-transferase fusion protein and activated with p38/RK MAP KINASE by Mr A.Clifton (University of Dundee) as described previously (Ben-Levy et al., 1995).

Replace the paragraph on page 51, lines 24-28, with the following new paragraph:

Monoclonal antibodies recognising the haemagglutonin (HA) epitope sequence YPYDVPDYA (SEQ ID NO:32), Protein G-Sepharose and histone H2B were obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were purified from rabbit skeletal muscle and rat liver respectively.

Replace the paragraph on page 52, lines 34-40, with the following new paragraph:

Assay of immunoprecipitated PKBα and protein determinations. Three aliquots of each immunoprecipitate (each comprising only 5% of the total immunoprecipitated PKBα) were assayed for PKBα activity towards the peptide

GRPRTSSFAEG (SEQ ID NO:1) as described previously (Cross et al., 1995). One unit of activity was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min. Protein concentrations were determined by the method of Bradford, 1976.

Replace the paragraph on page 55, lines 18-23, with the following new paragraph:

All peptides used to assay PKBα, and TTYADFIASGRTGRRNAIHD (SEQ ID NO:33)(the specific peptide inhibitor of cyclic AMP dependent protein kinase - PKI) were synthesised on an Applied Biosystems 431A peptide synthesizer. Their purity (> 95%) was established by HPLC and electrospray mass spectrometry, and their concentrations were determined by quantitative amino acid analysis.

Replace the paragraph on page 55, line 25 through page 56, line 32, with the following new paragraph:

Preparation and assay of PKBα. The construction of cytomegalovirus vectors (pCMV5) of the human haemagglutonin epitope-tagged wild type (HA-PKBα) was described previously (Alessi et al., 1996). 293 cells grown on 10 cm dishes were transfected with a DNA construct expressing HA-PKBα using a modified calcium phosphate procedure (Alessi et al., 1996). The cells were deprived of serum for 16h prior to lysis and, where indicated, were stimulated for 10 min in the presence of 50 ng/ml IGF-1 to activate PKBα. The cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μM Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate centrifuged at 4°C for 10 min at 13,000 x g and the supernatant obtained from one 10 cm dish of cells (2-3 mg protein) was incubated for 60 min

on a shaking platform with 20 μl of protein G-Sepharose coupled to 10 μg of HA monoclonal antibody. The suspension was centrifuged for 1 min at 13,000 x g, the Protein G-Sepharose-antibody-HA-PKBα complex washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by vol) 2-mercaptoethanol). The PKBα immunoprecipitates were diluted in Buffer B to an activity of 2.0 U/ml towards the Crosstide peptide GRPRTSSFAEG (SEQ ID NO:1) and 0.1 ml aliquots snap frozen in liquid nitrogen and stored at -80°C. No significant loss of PKBα activity occurred upon thawing the PKBα immunoprecipitates or during storage at -80°C for up to 3 months. The standard PKBα assay (50 μl) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, 0.2 U/ml PKBα, Crosstide (30 μM), 10 mM magnesium acetate and 0.1 mM [γ^{32}P]ATP (100-200 cpm/pmol). The assays were carried out for 15 min at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described (Alessi et al., 1995). One unit of activity was that amount of enzyme which catalysed the phosphorylation of 1 nmol of Crosstide in 1 min. The phosphorylation of other peptides, histone H2B and MBP were carried out in an identical manner. All the Crosstide activity in HA-PKBα immunoprecipitates is catalysed by PKBα (see Results) and the PKBα concentration in the immunoprecipitates was estimated by densitometric scanning of Coomassie blue-stained polyacrylamide gels, using bovine serum albumin as a standard. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (Bradford et al., 1976). Michaelis constants (Km) and Vmax values were determined from double reciprocal plots of 1/V against 1/S, where V is the initial rate of